Use of conventional taxonomy, electrophoretic karyotyping and DNA–DNA hybridization for the classification of fermentative apiculate yeasts

Ann Vaughan-Martini, Paola Angelini and Gianluigi Cardinali

Author for correspondence: Ann Vaughan-Martini. Tel: +39 075 585 6479. Fax: +39 075 585 6470.
e-mail: avaughan@unipg.it

Industrial Yeasts Collection (DBVPG), Dipartimento di Biologia Vegetale, Università di Perugia, Sez. Microbiologia Applicata, Borgo XX Giugno 74, 06121 Perugia, Italy

A taxonomic study was conducted that considered strains of the genera *Hanseniaspora*/*Kloeckera* held in the Industrial Yeasts Collection (DBVPG) of the Dipartimento di Biologia Vegetale of the Università di Perugia, Italy. Standard phenotypic as well as molecular criteria were considered in an effort to revisit the classification of these strains, some of which have been in the collection for about 50 years. Results of salient physiological tests showed that some of the DBVPG and type strains could not be identified by current taxonomic keys. Electrophoretic karyotypes were identical for some species, with the type strains of the seven accepted species showing only five distinct chromosomal patterns. DNA–DNA hybridization analyses, using a non-radioactive dot-blot technique, allowed for the distinction of taxa. The taxonomic implications of these results are discussed.

Keywords: *Hanseniaspora*, conventional and molecular taxonomy, non-radioactive dot-blot DNA reassociation, pulsed-field gel electrophoresis

INTRODUCTION

The genus *Hanseniaspora* was created when Klöcker (1912) described the species *Hanseniaspora valbyensis*, although studies of fermenting, apiculate yeasts began much earlier, in 1870, when Reess designated a non-sporulating strain as *Saccharomyces apiculatus* (Reess, 1870). The years that followed saw many different treatments of the classification and nomenclature of this group of yeasts due to problems experienced by investigators in the perception of a sexual cycle.

In spite of the absence of sporulation, Reess (1870) probably included *S. apiculatus* in a perfect genus because of its ability to ferment grape must, a trait far from universal in yeasts. It would not be until 28 years later that Beijerinck (1898) observed sporulation, with a direct transformation of the vegetative cell into an ascus containing, according to the author, between four and six ascospores. At that time, it was also pointed out that *S. apiculatus* tended to lose sexual capacity with prolonged cultivation. Lindner (1903) also observed a sexual cycle for this species and designated the genus *Hansenia*, for apiculate yeasts that form spores. Zikes (1911), however, was unable to

confirm sporulation in some strains of *Hansenia* and therefore assigned it to an anamorphic taxon of fermenting, apiculate yeasts. He then proposed the generic name *Hanseniaspora* for the perfect taxa. Finally, after a series of incongruences in nomenclature, *Hansenia* was replaced by the generic name *Kloeckeria* and then *Kloeckera* (Janke, 1928).

Over the years, additional apiculate yeasts were described and *Hanseniaspora* has grown from the original single species described in the first edition of the monograph *The Yeasts: a Taxonomic Study* (Lodder & Kreger-van Rij, 1952) to the present six recognized taxa in the fourth edition (Smith, 1998a). Over the same time-period, the genus *Kloeckera* has been reduced to a single species, *Kloeckera lindneri* (Kloecker) Janke (Janke, 1928; Smith, 1998b), since studies of molecular taxonomy have demonstrated a high genomic relationship between all of the teleomorphic and anamorphic forms (Smith *et al.*, 1977; Meyer *et al.*, 1978) except for *Kloeckera lindneri*.

The present study set out to reinvestigate some strains of the DBVPG Industrial Yeasts Collection classified as the most common species of *Hanseniaspora*/*Kloeckera* involved in grape-must fermentation in Italy: *Hanseniaspora guilliermondii* with its imperfect form *Kloeckera apis*, *Hanseniaspora osmophila* (ana-

Abbreviation: PFGE, pulsed-field gel electrophoresis.

morphs *Kloeckera corticis* and *Kloeckera magna*), *Hanseniaspora uvarum* (*Kloeckera apiculata*) and *Hanseniaspora vineae* with *Kloeckera africana*. The DBVPG collection holds over 150 strains belonging to this group that have been isolated from various substrates over a period of about 50 years. Since the separation of these taxa is sometimes difficult, especially in the absence of a sexual cycle, it was deemed necessary to confirm the original designations of some of these strains by employing the most recent conventional criteria (Smith, 1998a, b) together with methods of molecular taxonomy.

METHODS

Organisms. The strains studied are listed in Table 1. Type strains of the perfect and imperfect species were kindly provided by the yeast division of the Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands.

Physiological studies. Growth responses on sole carbon and nitrogen sources, as well as resistance to cycloheximide (actidione), were determined in liquid medium on a roller drum at 25 °C by using the methods described by Yarrow (1998). Maximum growth temperatures were determined by using static cultures containing YEPG (1% yeast extract,

1% peptone, 2% glucose) equilibrated in a water bath. An effort to induce a sexual cycle was made following current recommendations (Smith, 1998a).

Preparation and analysis of nuclear DNA

Pulsed-field gel electrophoresis (PFGE). Intact chromosomal DNA for PFGE was prepared as reported previously (Cardinali *et al.*, 1995). Gels composed of 1% agarose (type II-A, medium EEO; Sigma) were analysed using a CHEF Mapper or DRII (Bio-Rad) with 0.5×TBE buffer. A temperature of 12–15 °C was maintained throughout the runs and the parameters of the electrophoresis are described in the figure legends.

Hybridization experiments. DNA extraction and hydroxyapatite (HTP; Bio-Rad) purification were performed according to the protocol proposed by Cardinali *et al.* (2000). DNA–DNA hybridization experiments were performed using a dot-blot procedure where probe DNA was labelled using the ECL-Direct non-radioactive procedure (Amersham) following the manufacturer's instructions. Pre-hybridization, hybridization and high-stringency washes (0.5% NaCl) were all carried out at 42 °C.

Densitometric analysis of the DNA–DNA hybridizations. X-ray film (Kodak X-OMAT; Sigma) was exposed for 1, 2, 5 or 15 min according to the intensity of the signal. Only non-saturated films were subjected to further analysis.

Table 1. DBVPG isolates examined in the present study

Strain	Species or original epithet	Source	Date of deposit
3062	<i>Hanseniaspora apuliensis</i> Castelli	Grape must; Israel	1950
3094	<i>Kloeckera apiculata</i>	Wine; Trentino, Italy	1951
3096	<i>Kloeckera magna</i>	Sweet filtered wine; Brindisi, Italy	1951
3272	<i>Kloeckera apiculata</i>	Grape must; Calabria, Italy	1952
3280	<i>Kloeckera apiculata</i>	Soil; The Netherlands	1952
3298	<i>Kloeckera africana</i>	Wine; Capoterra (Sardinia), Italy	1952
3447	<i>Kloeckera apiculata</i>	Milk; Perugia, Italy	1955
3539	<i>Kloeckera apiculata</i>	Flowers; Perugia, Italy	1955
3542	<i>Kloeckera apiculata</i>	Soil; Puerto Lapiche, Spain	1955
3543	<i>Kloeckera magna</i>	Grapes; Cerro del Moro (La Mancha), Spain	1955
3582	<i>Kloeckera apiculata</i>	Grapes; Fuen Mayor (Rioja), Spain	1956
4033	<i>Kloeckera magna</i>	Cavern; Italy	1963
4059	<i>Kloeckera magna</i>	Grape must; Slovenia	1968
4159	<i>Kloeckera magna</i>	Grape must; Slovenia	1968
4160	<i>Kloeckera magna</i>	Grape must; Slovenia	1968
4351	<i>Kloeckera apiculata</i>	Sea water; Italy	1994
6717 ^T	<i>Pseudosaccharomyces apiculatus</i> (Reess) Klöcker	Unknown	1993
6718 ^T	<i>Hanseniaspora uvarum</i> (Niehaus) Shehata <i>et al.</i>	Muscetelle grapes; Ukraine	1990
6774 ^T	<i>Hanseniaspora valbyensis</i> Klöcker	Soil; Denmark	1993
6790 ^T	<i>Kloeckera apis</i> Lavie ex M. T. Smith <i>et al.</i>	Trachea of bee; France	1993
6791 ^T	<i>Kloeckera corticis</i> (Klöcker) Janke	Bark of tree; Denmark	1993
6792 ^T	<i>Kloeckera africana</i> (Klöcker) Janke	Soil; Algeria	1993
6793 ^T	<i>Kloeckera japonica</i> Saito & Otani	Sap of tree; Japan	1993
6794 ^T	<i>Pseudosaccharomyces javanicus</i> Klöcker	Soil; Java	1993
6795 ^T	<i>Hanseniaspora osmophila</i> (Niehaus) Phaff <i>et al.</i> ex M. Th. Smith	Ripe Reisling grapes; Germany	1993
6796 ^T	<i>Hanseniaspora guilliermondii</i> Pijper	Infected nail; South Africa	1993
6797 ^T	<i>Hanseniaspora vineae</i> van der Walt & Tscheuschner	Soil of vineyard; South Africa	1993
6798 ^T	<i>Hanseniaspora occidentalis</i> M. T. Smith	Soil; West Indies	1993
7132 ^T	<i>Kloeckera lindneri</i> (Klöcker) Janke	Soil; Java	1998

A calibration curve was obtained by plotting the values of serial probe dilutions against the corresponding signals expressed as grey levels (according to a 256-level system). A regression equation was used to calculate the reassociation level of each sample. Video densitometric analysis was carried out using the free-domain software NIH IMAGE 1.62b and densitometric data were processed with KALEIDA GRAPH (Synergy Software).

RESULTS AND DISCUSSION

Physiological reactions

A summary of salient phenotypic characteristics (Table 2) shows that most of the DBVPG strains

repeated the assimilative patterns observed at the time of the original species designation. According to the most recent criteria for the classification of the genera *Hanseniaspora*/*Kloeckera* (Smith, 1998a, b), all strains originally classified as *Kloeckera apiculata*, as well as the type strains of *Hanseniaspora guilliermondii*/*Kloeckera apis* and *Hanseniaspora uvarum*/*Kloeckera apiculata*, were unable to utilize sucrose or maltose as sole carbon sources, whereas those designated as *Kloeckera africana* or *Kloeckera magna*, together with the type strains of *Kloeckera africana* (6792^T) and *Kloeckera corticis* (6791^T), showed positive results on one or both of those sugars. On the other hand, the type strains of *Hanseniaspora osmophila* (6795^T) and

Table 2. Comparison of physiological reactions and electrophoretic profiles of selected strains of *Hanseniaspora*/*Kloeckera*

Strain	Assimilation*			Growth				Apparent phenotype‡	
	MAL	SUC	2KG	100 ACT†	30 °C	35 °C	37 °C	Physiology	CHEF§
3062	—	—	+	+	+	+	+	G	G/U
3094	—	—	+	+	+	—	—	U	G/U
3096	+	—	+	—	+	—	—	Os/U	Os/Vi
3272	—	—	+	+	+	—	—	U	G/U
3280	—	—	+	+	+	—	—	U	G/U
3298	+	±	—	—	+	—	—	Os	Os/Vi
3447	—	—	+	ND	+	+	—	U	G/U
3539	—	—	+	ND	+	—	—	U	G/U
3542	—	—	+	ND	+	—	—	U	G/U
3543	±	±	—	—	+	—	—	Os	Os/Vi
3582	—	—	+	+	+	—	—	U	G/U
4033	+	±	—	—	+	—	—	Os	Os/Vi
4059	+	±	+	ND	+	—	—	Os/U	Os/Vi
4159	+	±	—	ND	+	+	+	G/Vi	Os/Vi
4160	+	—	—	ND	+	—	—	Os	Os/Vi
4351	—	—	+	+	+	—	—	U	G/U
6717 ^T	—	—	+	+	—	—	—	U/Va	G/U
6718 ^T	—	—	+	+	+	—	—	U	G/U
6774 ^T	—	—	—	+	+	—	—	L/Va	Va
6790 ^T	—	—	+	+	+	+	+	G	G/U
6791 ^T	+	—	—	—	+	—	—	Os	Os/Vi
6792 ^T	+	±	—	—	+	+	—	Vi	Os/Vi
6793 ^T	—	—	—	+	+	—	—	L	Va
6794 ^T	—	+	—	ND	+	—	—	Oc	Oc
6795 ^T	—	—	+	—	+	—	—	?	Os/Vi
6796 ^T	—	—	+	+	+	+	+	G	G/U
6797 ^T	—	—	—	—	+	+	+	?	Os/Vi
6798 ^T	—	+	—	ND	+	—	—	Oc	Oc
7132 ^T	—	—	—	ND	—	—	—	Va?	L

* Assimilation of maltose (MAL), sucrose (SUC) and 2-ketogluconate (2KG) was tested.

† Resistance to 100 p.p.m. cycloheximide (actidione).

‡ Phenotypes are indicated as G (*Hanseniaspora guilliermondii*), L (*Kloeckera lindneri*), Oc (*Hanseniaspora occidentalis*), Os (*Hanseniaspora osmophila*), U (*Hanseniaspora uvarum*), Va (*Hanseniaspora valbyensis*) and Vi (*Hanseniaspora vineae*).

§ Electrophoretic karyotype obtained by PFGE.

ND, Not done.

Hanseniaspora vineae (6797^T) were not able to use either sugar, even though, according to current keys, maltose assimilation should be positive for these species (Smith, 1998a). Although all strains of *Hanseniaspora guilliermondii*/*Kloeckera apis* and *Hanseniaspora uvarum*/*Kloeckera apiculata* showed growth on 2-ketogluconate as expected, three other strains originally designated as *Hanseniaspora osmophila* or *Kloeckera magna* (3096, 4059 and 6795^T) were also unexpectedly able to utilize this carbon source. Finally, temperature reactions at 37 and 30 °C did not always correspond to current taxonomic indications (Smith, 1998a, b). As a consequence of these physiological variations, some of the type and DBVPG strains had to be assigned an 'apparent phenotype' (Table 2) that was different from the original species designation.

The physiological inconsistencies observed in this study have also been encountered by previous authors. Even though Meyer and coworkers divided *Hanseniaspora valbyensis* into two separate species (Meyer *et al.*, 1977), with strains of *Hanseniaspora guilliermondii* characterized by the formation of four spores per ascus and the ability to grow at 37 °C and to assimilate 2-ketogluconate as a sole carbon source, Phaff (1970) had already noted that some four-spored strains gave negative reactions to some of these tests. These observations, together with the results of this and another study of strains of *Hanseniaspora*/*Kloeckera* (Boekhout *et al.*, 1994), renew the reservations regarding the validity of physiological criteria as a basis for species designations. One could speculate either that these reactions are not universal for all strains of a species or that they are not stable over time. It is well known that the shortcomings of the use of physiological tests for the distinction of taxa derive from the fact that many of the systems used for the long-term storage of strains can lead to point mutations (Scheda & Yarrow, 1966, 1968; Stahl & Esser, 1979a, b). Since the nucleotide base sequences that make up the genes that control many of these reactions often represent a minimal part of the genome (Price *et al.*, 1978), minor modifications could result in significant changes in species designations based on these criteria. For this reason, in the last 30 years, taxonomic studies have been integrated with tests based on more stable parameters such as the comparison of cellular macromolecules, particularly DNA and RNA (Kurtzman & Phaff, 1987; Phaff, 1989; James *et al.*, 1997; Kurtzman & Robnett, 1998).

Electrophoretic karyotypes

The comparison of the electrophoretic karyotypes of unknown cultures against those of type strains can sometimes give a confirmation of identifications obtained by conventional taxonomic methods (Vaughan-Martini *et al.*, 1999). However, in this investigation, it was seen that this type of analysis cannot always be useful for the distinction of strains belonging to the genera *Hanseniaspora*/*Kloeckera*, as

Fig. 1. Electrophoretic karyotypes of the type strains of *Hanseniaspora* and *Kloeckera*. Lanes: S, *Pichia canadensis* standard (referred to as *Hansenula wingei*), obtained from Bio-Rad; 1, *Kloeckera lindneri* DBVPG 7132^T; 2, *Hanseniaspora occidentalis* DBVPG 6798^T; 3, *Kloeckera javanicus* DBVPG 6794^T; 4, *Hanseniaspora vineae* DBVPG 6797^T; 5, *Kloeckera africana* DBVPG 6792^T; 6, *Hanseniaspora osmophila* DBVPG 6795^T; 7, *Kloeckera corticis* DBVPG 6791^T; 8, *Kloeckera japonica* DBVPG 6793^T; 9, *Hanseniaspora valbyensis* DBVPG 6774^T; 10, *Hanseniaspora guilliermondii* DBVPG 6796^T; 11, *Kloeckera apis* DBVPG 6790^T; 12, *Hanseniaspora uvarum* DBVPG 6718^T; 13, *Kloeckera apiculata* DBVPG 6717^T. Electrophoresis conditions: CHEF DRII; 72 h; 60 → 420 s switch, 4.5 V cm⁻¹.

only five distinct profiles were observed for the type strains of the seven currently accepted species (Fig. 1). Two chromosomal patterns were each shared by two species: the karyotype of *Hanseniaspora guilliermondii* was identical to that of *Hanseniaspora uvarum* (strains 6796^T, 6790^T, 6718^T and 6717^T; lanes 10–13), while *Hanseniaspora osmophila* and *Hanseniaspora vineae* (strains 6797^T, 6792^T, 6795^T and 6791^T; lanes 4–7) also had the same profiles. These similarities could not be overcome even when the conditions of electrophoresis were modified radically (as shown in Figs 2 and 3). Distinctive profiles were seen only for the type strain of *Kloeckera lindneri* (7132^T; Fig. 1, lane 1) and for the perfect/imperfect pairs *Hanseniaspora valbyensis*/*Kloeckera japonica* (6793^T and 6774^T; Fig. 1, lanes 8 and 9) and *Hanseniaspora occidentalis*/*Kloeckera javanica* (6798^T and 6794^T; Fig. 1, lanes 2 and 3). Nevertheless, the apparent affinities observed between the species *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* or between *Hanseniaspora osmophila* and *Hanseniaspora vineae* are not in agreement with extensive studies of DNA reassociation, which demonstrated the presence of six distinct species in *Hanseniaspora* with reciprocal base sequence differences normally exceeding 80% (Meyer *et al.*, 1977, 1978; Smith *et al.*, 1977). These separations were also confirmed by the DNA hybridizations performed in this study (Table 3), as well as by three separate investigations considering 26S rDNA sequences (Yamada *et al.*, 1992a; Boekhout *et al.*, 1994; Kurtzman & Robnett, 1998).

Table 3. Comparative percentage DNA–DNA hybridization among the type strains of the genera *Hanseniaspora*/*Kloeckera*

Values in bold were obtained in the present study. Other values were taken from Smith *et al.* (1977) and Meyer *et al.* (1978) and represent measurements using the same strains or, in the case of values in square brackets, different strains of the same species. *H.*, *Hanseniaspora*; *K.*, *Kloeckera*.

Strain	Source of probe DNA						
	2	3	5	8	10	11	13
1. <i>H. uvarum</i> 6717*	90 , 100	32 , [11]	20 , 14	4	3	18	0
2. <i>H. uvarum</i> 6718 ^T	100 , 100	33 , [15]	14 , 10	4 , [15]	0 , [5]	15 , [10]	0
3. <i>H. guilliermondii</i> 6790*	4 , 30	100 , 100	14 , 11	0	2	17	0
4. <i>H. guilliermondii</i> 6796 ^T	10 , 27	81 , 100	26 , 19	1	0	25	0
5. <i>H. valbyensis</i> 6774 ^T	22 , 20	23 , [13]	100 , 100	5	4	12	0
6. <i>H. valbyensis</i> 6793*	11 , 27	24 , [10]	68 , 100	8	6	9	0
7. <i>H. osmophila</i> 6791*	9 , 1	18	11 , [12]	98 , 85	23 , 56	12 , [15]	0
8. <i>H. osmophila</i> 6795 ^T	15 , 13	20	18	100 , 100	18 , 60	17	0
9. <i>H. vineae</i> 6792*	9	18	15 , [5]	36 , 46	99 , 93	20 , [8]	0
10. <i>H. vineae</i> 6797 ^T	0 , 10	20 , [12]	12 , [13]	35 , [38]	100 , 100	12 , [11]	0
11. <i>H. occidentalis</i> 6798 ^T	<1	15 , [7]	10 , 11	6 , [16]	0	100 , [82]	0
12. <i>H. occidentalis</i> 6794*	2	15 , [14]	25 , [9]	14 , 19	21	74 , [100]	0
13. <i>K. lindneri</i> 7132 ^T	<1	0	9	0	0	0	100

* Type strain of the corresponding anamorph.

Fig. 2. Electrophoretic karyotypes of selected DBVPG strains related to *Hanseniaspora guilliermondii* or *Hanseniaspora uvarum*. Lanes: S, *Pichia canadensis* standard (referred to as *Hansenula wingei*), obtained from Bio-Rad; 1–6, *Hanseniaspora uvarum* strains DBVPG 4351, DBVPG 3542, DBVPG 3539, DBVPG 3272, DBVPG 3094 and DBVPG 6718^T; 7, *Kloeckera apiculata* DBVPG 6717^T; 8, *Hanseniaspora guilliermondii* DBVPG 6796^T; 9, *Kloeckera apis* DBVPG 6790^T; 10, *Hanseniaspora uvarum* DBVPG 3582; 11, *Hanseniaspora uvarum* DBVPG 3280; 12, *Hanseniaspora guilliermondii* DBVPG 3062; 13, *Hanseniaspora uvarum* DBVPG 3447. Electrophoresis conditions: CHEF Mapper; 72 h; 120° angle; 60 → 420 s switch, 4.5 V cm⁻¹.

The presence of similar or even identical electrophoretic karyotypes for different species is not new. A well-documented example can be seen in the four taxa of the *sensu stricto* complex of the genus *Saccharo-*

Fig. 3. Electrophoretic karyotypes of selected DBVPG strains related to *Hanseniaspora osmophila* or *Hanseniaspora vineae*. Lanes: S1, *Saccharomyces cerevisiae* standard; 1, *Hanseniaspora vineae* DBVPG 6797^T; 2, *Kloeckera africana* DBVPG 6792^T; 3–5, *Hanseniaspora osmophila* strains DBVPG 4033, DBVPG 4159 and DBVPG 3543; 6, *Hanseniaspora vineae* DBVPG 4160; 7, *Hanseniaspora osmophila* DBVPG 4059; 8, *Hanseniaspora vineae* DBVPG 3298; 9, *Hanseniaspora vineae* DBVPG 3096; 10, *Kloeckera corticis* DBVPG 6791^T; 11, *Hanseniaspora osmophila* DBVPG 6795^T; 12, *Hanseniaspora vineae* DBVPG 6797^T; 13, *Kloeckera africana* DBVPG 6792^T; S2, *Pichia canadensis* standard (referred to as *Hansenula wingei*). Standards were obtained from Bio-Rad. Electrophoresis conditions: CHEF DR11; 56 h; 500 s switch, 3 V cm⁻¹.

myces, where it is difficult to differentiate species solely on the basis of chromosomal profiles (Vaughan-Martini *et al.*, 1993). As a result, additional molecular analysis such as DNA–DNA hybridization is necessary for a clear distinction of taxa.

When the electrophoretic karyotypes of the DBVPG strains under study were compared with the patterns obtained for the type strains (Figs 2 and 3), it was not

Table 4. Percentage DNA–DNA hybridization between type and other strains of the genera *Hanseniaspora*/*Kloeckera*

For explanations of the different phenotypes, see footnote to Table 2. Values over 70 % are in bold. *H.*, *Hanseniaspora*; *K.*, *Kloeckera*.

Strain	Phenotype		Source of probe DNA						
	Physiology	CHEF	<i>H. uvarum</i> 6718 ^T	<i>H. guilliermondii</i> 6790*	<i>H. valbyensis</i> 6774 ^T	<i>H. osmophila</i> 6795 ^T	<i>H. vineae</i> 6797 ^T	<i>H. occidentalis</i> 6798 ^T	<i>K. lindneri</i> 7132 ^T
3062	G	G/U	16	98	30	2	3	19	0
3094	U	G/U	98	35	16	7	22	21	0
3096	Os/U	Os/Vi	5	24	14	36	89	42	0
3272	U	G/U	92	35	11	2	0	12	0
3280	U	G/U	86	30	10	4	17	22	0
3298	Os	Os/Vi	6	26	7	3	93	42	0
3447	U	G/U	84	27	32	0	0	26	0
3539	U	G/U	98	33	40	2	10	25	0
3542	U	G/U	88	31	37	9	12	18	0
3543	Os	Os/Vi	13	20	20	97	20	27	0
3582	U	G/U	88	21	9	5	15	28	0
4033	Os	Os/Vi	19	20	17	77	32	28	0
4059	Os/U	Os/Vi	7	25	12	74	37	30	0
4159	G/Vi	Os/Vi	11	18	17	85	30	25	0
4160	Os	Os/Vi	6	19	10	3	98	28	0
4351	U	G/U	97	33	28	2	5	12	0

possible to assign any strain to a single species on the basis of PFGE. As in the case of the type strains, the profiles of those cultures originally designated as *Hanseniaspora apuliensis* or *Kloeckera apiculata* corresponded to those of the *Hanseniaspora guilliermondii*–*Hanseniaspora uvarum* group (Fig. 2), whereas those classified as *Kloeckera africana* or *Kloeckera magna* were similar to both *Hanseniaspora osmophila* and *Hanseniaspora vineae* (Fig. 3). As a consequence, those strains with karyotypes similar to the species pairs *Hanseniaspora guilliermondii*/*Hanseniaspora uvarum* or *Hanseniaspora osmophila*/*Hanseniaspora vineae* had to be assigned a double ‘CHEF phenotype’ (Table 2).

In spite of these limitations, the electrophoretic karyotypes observed for those strains that gave unexpected physiological reactions (Figs 2 and 3, Table 2) consistently showed high similarities to those of the species to which they were originally assigned, rather than confirming the ‘apparent phenotype’ deduced from the physiological reactions performed in this study. This could therefore confirm the observations that phenotypic results can change as a result of genomic mutations caused during prolonged maintenance in culture collections where low-temperature conservation techniques are not employed (Scheda & Yarrow, 1966, 1968; Stahl & Esser, 1979a, b; Kirsop & Kurtzman, 1988).

DNA–DNA reassociation

As a validation of the non-radioactive dot-blot hybridization method used in this study, our results

were compared to those obtained in previous investigations (Smith *et al.*, 1977; Meyer *et al.*, 1978), which employed the radioactive membrane filter technique (Denhardt, 1966). As seen in Table 3, there was good agreement between the results of this and the earlier studies. The only exceptions were seen for *Hanseniaspora vineae* (6797^T) and the type strains of *Hanseniaspora osmophila* (6791^T and 6795^T), where the percentage homology observed in this study was less than half that observed by Meyer and coworkers (23 and 18 as opposed to 56 and 60 %).

The results of the DNA–DNA hybridization experiments between the DBVPG strains and all type strains (Table 4) partially confirmed the phenotypes established on the basis of physiological and/or CHEF data (Table 2). In particular, when chromosomal profiles alone were considered, strains with a double ‘CHEF phenotype’ proved to be highly related (74–100 %) to only one of the two species, while showing significantly lower or no DNA reassociation with any other strain (values normally below 40 %).

The results of this investigation also find confirmation in two studies that compared the 26S rDNA sequences of apiculate yeasts (Yamada *et al.*, 1992a; Boekhout *et al.*, 1994), where a clear separation of all currently accepted species of *Hanseniaspora* Smith as well as of *Kloeckera lindneri* was shown. Interestingly, those species that, according to Boekhout *et al.* (1994), have the closest affinities (expressed as numbers of differences in the D1/D2 region of the large subunit of rDNA) were also those that showed identical karyo-

types (Figs 1–3). *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* differ by six nucleotides, while *Hanseniaspora osmophila* and *Hanseniaspora vineae* show seven differences. On the contrary, differences above nine nucleotides, as seen between *Hanseniaspora valbyensis* and *Kloeckera lindneri*, corresponded to significantly different chromosomal patterns. A similar situation exists within the genus *Saccharomyces*, where the strains involved in alcoholic fermentation (the *sensu stricto* complex) have both very similar electrophoretic karyotypes (Cardinali & Martini, 1994) and the closest intragenetic relationships as seen by the extent of nucleotide substitutions in rRNA sequences (Kurtzman & Robnett, 1991).

Yamada *et al.* (1992b) proposed the division of the genus *Hanseniaspora* on the basis of partial rRNA sequences and other phenotypic differences. *Hanseniaspora valbyensis*, *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* would be maintained, while the genus *Kloeckeraspora* (Janke, 1928) would be revived for *Hanseniaspora occidentalis*, *Hanseniaspora osmophila* and *Hanseniaspora vineae* (Yamada *et al.*, 1992b). Although they recognized a certain distance between these two groups of species, Boekhout *et al.* (1994) did not agree with this modification in the light of the intraspecific variability present within these groups. According to those authors, a division could introduce too much confusion for species distinction based upon phenotypic criteria. In view of the variations in physiological profiles observed in this study, we also disagree with excessive generic separations. Finally, the unity of the genus *Hanseniaspora* was confirmed in an extensive comparison of the rDNA large subunit D1/D2 domains of over 500 currently accepted yeast species (Kurtzman & Robnett, 1998). In this latter investigation, it was shown that species of *Hanseniaspora*, together with *Kloeckera lindneri*, form a distinct subclade within the *Saccharomyces* clade.

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